

# XXX. ON THE COMPOSITION OF THE UNSAPONIFIABLE MATTER OF THE ETHER EXTRACT OF HUMAN FAECES.

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IN a series of papers [1907–1912] on the origin and destiny of cholesterol in the animal organism, it was shown that cholesterol is never excreted as such, or in any recognisable reduced form, in the *normal* faeces of herbivorous animals, such as horses, cattle, sheep and rabbits. Evidence was also brought forward which lent support to the view that in herbivora cholesterol is a substance which is strictly conserved in the animal economy, that when the destruction of the red blood corpuscles, and possibly other cells, takes place in the liver, their cholesterol is excreted in the bile and that the cholesterol of the bile is re-absorbed in the intestine along with the bile salts, finding its way into the blood stream to be used again in cell anabolism; and, further, that any waste of cholesterol might be made up from that taken in with the food [Dorée and Gardner, 1909]. This latter process would be limited in herbivorous animals by the fact that their normal food does not contain cholesterol, but isomeric substances, such as phytosterols, which presumably would have to be converted into cholesterol before utilisation [Fraser and Gardner, 1910]. The faeces of herbivora, however, contain a considerable amount of non-crystalline unsaponifiable matter, consisting mainly of a reddish oil. The dry faeces of cows gave, after separating any crystalline matter, 0.39 % and of sheep 0.4 % of a red oil. 3 kilos. of dry summer faeces of wild rabbits yielded 0.21 % of so-called hippocoprosterol and 0.77 % of oil [Dorée and Gardner, 1908, 1].

It was found that this oil (from rabbits) was very stable and could be fractionally distilled at a pressure of under 1 mm. without any decomposition, though the process was tedious and difficult. Four main fractions were obtained at the following temperatures: (1) 98°, (2) 164–168°, (3) 215–220°, (4) 260–265°. In the flask there remained a transparent brittle resinous substance, which was still distilling without a trace of decomposition at the softening point of glass.

Fraction (1) was a pale yellow fairly mobile oil, with a smell recalling that of pine oil, which contained 82.13 % C and 11.96 % H. On treating a small quantity in chloroform solution with excess of acetic anhydride and a few drops of strong sulphuric acid, it gave a deep reddish brown colour, which gradually changed to a dirty brownish green. Fraction (2) had a faint pine odour and gave a similar reaction. Fraction (3) was a thick oil which gave the cholesterol colour reactions with the above reagents in a modified manner. Fraction (4)—the largest fraction—was at ordinary temperatures a clear yellow glass, which remained perfectly transparent after standing for ten years. It gave the sterol colour reactions with acetic anhydride and sulphuric acid in a well-marked though modified manner. Combustion showed that it had nearly the same composition as cholesterol.

	% C	% H
	84.21	11.56
	84.05	11.99
Calculated for $C_{27}H_{46}O$ ...	83.94	11.92

All attempts to prepare a crystalline acetate or benzoate failed.

The faeces of rabbits fed on ether-extracted grass also contained similar oils, but quantitative experiments to show whether in less quantity were not made.

Oils which gave a dirty brown or brownish green colour with acetic anhydride and sulphuric acid were also found in the unsaponifiable matter of the ether extracts of grass and other plants.

In carnivora, such as dog [Dorée and Gardner, 1908, 2] and cat [Ellis and Gardner, 1909], provided the body weight remains constant, the cholesterol excreted in the faeces can be all, or nearly all, accounted for by that naturally ingested with the food. Klein [1910], in his experiments, also arrived at a similar conclusion. In adult man, under normal conditions, cholesterol is not excreted as such in the faeces, but always in the form of one of the bi-hydrocholesterols. The main substance is coprosterol, but a certain amount of the isomeric  $\beta$ -cholesterol is also present.

Ellis and Gardner [1912] showed that the quantity of coprosterol found in the faeces of man can be largely accounted for by the cholesterol taken in with the food, provided the body weight remains constant. If, however, a rapid loss of weight takes place, as in illness, the output of cholesterol may largely exceed the intake. More recent experiments carried out by more accurate methods than were previously available, the results of which will form the subject of another paper, have shown that there is normally a regular though very small excretion of coprosterol in excess of the cholesterol taken in with the food. This would suggest that some organ of the body is capable of synthesising cholesterol to make up the waste. The unsaponifiable matter of human faeces contains in addition to crystalline sterols a considerable amount of non-crystallisable oils. These oils are somewhat variable in quantity, and often considerably exceed the amount of crystalline matter. After separa-

tion of coprosterol, etc. by means of digitonin they usually give the Burchardt-Liebermann reaction, though the colours are often partially masked by the dirty colour of the material. This colour reaction does not necessarily indicate that the oily matter contains derivatives of the sterol group, as the reaction is given by a variety of other substances.

It seemed, however, important to submit the unsaponifiable matter of faeces to a more detailed chemical examination.

For this purpose a large quantity—over 1 cwt.—of mixed human faeces from a Yorkshire village was collected and dried partly by heat and partly by means of plaster of Paris. The dry material was ground up and extracted with ether in large metal extractors, arranged on the principle of the ordinary small Soxhlet's apparatus. The ethereal solution of the extract was saponified in the usual manner in the cold with a large excess of an alcoholic solution of sodium ethoxide. The precipitated soaps were filtered off and re-extracted with ether. The ethereal filtrates were then washed free from traces of soap and alcohol by repeatedly shaking with water. The ether was then distilled off and the oily unsaponifiable matter taken up in acetone. The acetone solution was allowed to crystallise fractionally and the process repeated several times in order to effect as complete a separation of crystalline matter from it as possible.

In view of the results mentioned above it seemed possible that both the crystalline and oily matter might be conveniently purified by distillation in superheated steam, or by distillation in a high vacuum, or a combination of both processes.

In order to test the validity of this method as far as possible, and to ascertain whether any intra-molecular change took place in the case of the crystalline sterols, the behaviour of the following substances was examined: cholesterol, coprosterol and  $\psi$ -coprosterol.

#### *Distillation of cholesterol.*

As it is well known that cholesterol can be distilled *in vacuo* unchanged, it was therefore only submitted to distillation in superheated steam.

Steam from a boiler was passed through a small spiral superheater and carried by a metal tube to the bottom of an ordinary distillation flask containing the cholesterol. This flask was well lagged and its delivery tube passed to the centre of a second distillation flask, which was connected with a long Liebig's condenser.

The cholesterol melted and then distilled over in the steam fairly rapidly. The greater portion condensed in the trap flask, where it remained in the molten condition, and the rest condensed in the condenser in the form of a solid white jelly-like emulsion. This solid emulsion never blocked the condenser, but from time to time partly under the influence of the pressure of the steam and partly of the running condensed water was forced from the condenser into the receiver in the form of candles. The emulsion could be readily

filtered and a good deal of the water separated by gentle pressure. The various distillates were recrystallised from alcohol. The small quantity of cholesterol remaining in the distillation flask was only slightly discoloured, and on recrystallisation from alcohol melted at 146–147°.

The cholesterol used melted at 147–148°, and had a specific rotary power  $[\alpha]_D = -36.39^\circ$ .

The fractions from the trap flask and the receiver after recrystallisation from alcohol both melted at 147–148°. They showed however a slight increase in rotation. The values determined were:

Undistilled cholesterol	...	...	...	-36.39°
Cholesterol from solid emulsion	...	...	...	-37.81°
Cholesterol from trap flask	...	...	...	-39.50°; another determination -39.46°
Cholesterol from residue in distilling flask	...	...	...	-35.59°.

The cholesterol from the trap flask was again recrystallised from 80 % alcohol and gave a value -41.4°.

The meaning of these variations has not been ascertained.

#### *Distillation of coprosterol.*

10 g. of fairly pure coprosterol were distilled at a pressure of about 1 mm. from a small retort heated by a metal bath. The material distilled over readily at 220–225°. The apparent temperature of distillation, however, appeared to depend to some extent on the temperature of the bath and the process was more of the nature of an evaporation than a true distillation. The distillate was very pale yellow in colour and solidified to a white mass. Only a faint trace of tarry matter remained in the retort. The distillate was crystallised from acetone. The main fraction on heating began to shrink together at 102° and ran in the tube at 107°. Another small crop was obtained on evaporating the mother liquors, which melted at 101–102°. The distilled coprosterol had a normal rotation and the acetate and benzoate normal properties. The following esters were also prepared and may be briefly described:

*Chloroacetate.* 5 g. coprosterol were dissolved in 10 cc. chloroform and warmed under a reflux with a slight excess of chloroacetyl chloride. The residue obtained on evaporating the solvent crystallised from ethyl acetate in needle-shaped crystals. On heating they began to soften at 143° and melted at 145–146°. The substance was only difficultly soluble in hot methyl and ethyl alcohols. It contained 7.93 % chlorine—theory 7.63, and its specific rotary power in chloroform was  $[\alpha]_D^{18} = +20.6^\circ$ .

*Bromoisovalerate.* This was prepared in a similar manner from bromoisovaleryl bromide. It crystallised readily from a mixture of ethyl acetate and alcohol, or from a mixture of chloroform and alcohol. It melted at 103–104° and had  $[\alpha]_D^{18} = +18.47^\circ$ . It contained 14.67 % bromine—theory 14.52.

*Stearate.* This was prepared by boiling coprosterol in chloroform solution with a slight excess of stearyl chloride. It was almost insoluble in hot alcohol,

but crystallised readily from ethyl acetate or acetone. It melted at 69–70°, and on cooling showed a faint yellowish green play of colours at the solidifying point.  $[\alpha]_D^{18} = +14.02^\circ$  (in chloroform).

*Analysis:* 82.21 % C, 12.46 % H; calculated for  $C_{45}H_{82}O_2$  82.82 % C, 12.27 % H.

*Palmitate.* This was prepared in a similar manner from palmityl chloride. It was only very slightly soluble in methyl alcohol, but could be readily crystallised from ethyl acetate or from acetone in glistening hexagonal plates. It melted at 65° and showed at its solidifying point a faint colour display—greenish blue, green and yellow.

*Laurate.* This was prepared from lauryl chloride in a similar manner. It was more soluble in alcohol than the homologous esters, and crystallised readily from ethyl acetate in glistening leaflets, which under the microscope appeared as flat hexagonal plates, rather like phytosterol in appearance. It softened at 58° and melted at 61–62°.

*Analysis:* 82.43 % C, 12.34 % H; calculated 82.11 % C, 12.28 % H.

On oxidation the distilled coprosterol gave the ketone coprostanone described by Dorée and Gardner [1908, 3] in normal manner.

The distillation of the coprosterol had evidently produced no change.

#### *Distillation of $\psi$ -coprosterol.*

The  $\psi$ -coprosterol was prepared in the usual manner by boiling coprosterol in amyl alcohol solution with sodium amylate, and subsequent elimination of unchanged coprosterol and  $\beta$ -cholestanol by digitonin. The  $\psi$ -coprosterol left in the filtrate from the insoluble digitonide was then purified by recrystallisation. In the mother liquors, after separating crystalline matter as completely as possible, there remained a small quantity of a stiff oil. This oil distilled in a vacuum of 1 mm. at about 210–220° without any decomposition. The distillate had a pale yellow colour and solidified to an amorphous glass, which remained perfectly transparent for several years. It gave the sterol colour reaction in chloroform solution with acetic anhydride and sulphuric acid in a well-marked manner.

*Analysis:* 83.97 % C, 11.65 % H; calculated for  $C_{27}H_{46}O$  83.94 % C, 11.92 % H.

It had therefore much the same composition as cholesterol or coprosterol.

The  $\psi$ -coprosterol was then distilled in superheated steam when it passed over in a similar manner to the isomer. After crystallisation it was dried and submitted to distillation in a vacuum of 1 mm. pressure. It evaporated over between 214–222° according to the temperature of the metal bath, leaving no residue. It solidified in the receiver almost at once. The solid was recrystallised from alcohol, acetone, light petroleum and finally from alcohol and ethyl acetate. On heating it began to shrink together at 120° and ran in the tube at 123° to a turbid liquid which cleared at 125°. On cooling it became cloudy

again and was quite solid at 120°. On remelting it behaved as before. This melting point is rather higher than that given by Dorée and Gardner [1908, 3] 119° and by Windaus [1916] 116–118°. It is difficult to say whether the distillation had resulted in higher purification, or some slight change had taken place. The rotary power was found to be  $[\alpha]_D^{14} = +28.84^\circ$ .

The constants hitherto recorded are: Dorée and Gardner + 31.55°, and Windaus + 31.62°. The esters did not crystallise so readily as those of coprosterol; a number were prepared, which had normal properties. The following, which we believe are new, are worth a brief description:

*$\psi$ -Coprosterol chloroacetate.* This was prepared in a similar manner to the coprosterol chloroacetate. It crystallised in glistening plates or flat needles. M.P. 68.5–69°. On analysis it gave Cl 7.82 %; calculated 7.91 %.

*$\psi$ -Coprosterol stearate* was prepared by heating one part  $\psi$ -coprosterol with 0.8 part stearyl chloride in chloroform solution. It was crystallised from a mixture of chloroform and alcohol, and also from ethyl acetate. M.P. 62–63°.  $[\alpha]_D^{18} = +28.89^\circ$  (in chloroform).

*Analysis:* 82.83 % C, 12.73 % H; calculated 82.57 % C, 12.53 % H.

*$\psi$ -Coprosterol laurate* was made in a similar manner from lauryl chloride. It was purified by crystallisation from a mixture of benzene and alcohol, melted at 38–39°, and had  $[\alpha]_D^{18} = +28.43^\circ$ .

*Analysis:* 82.43 % C, 12.23 % H; calculated 82.11 % C, 12.28 % H.

As distillation did not seem to have produced any material alteration in cholesterol and coprosterol, the crude crystalline matter was purified by distillation in superheated steam and subsequent distillation in a vacuum of 1 mm. or under. The white solid matter thus obtained was then subjected to an elaborate fractional crystallisation from acetone and from 80 % alcohol. The bulk proved to be pure coprosterol, various fractions melting between 100 and 108°. Smaller fractions were, however, obtained which melted at 111–112° and 112–113°. On repeatedly recrystallising these, a fraction was isolated which began to shrink together at 113° and melted at 115–116°.

Hans Fischer stated that the melting point of coprosterol could be raised to 112–115° by repeated crystallisation.

In order to find whether this was pure coprosterol or contained some isomer, it was heated in amyl alcohol solution with sodium amylate in the usual manner to convert coprosterol into  $\psi$ -coprosterol. The product was dissolved in alcohol, and an alcoholic solution of digitonin added, when a small quantity of an insoluble digitonide was precipitated. The bulk of the product remained in solution and yielded pure  $\psi$ -coprosterol. The insoluble digitonide was dissociated by heating in the vapour of boiling xylene, and after driving off the xylene in steam, a small quantity of crystalline sterol was obtained. This gave no colour reaction with acetic anhydride and sulphuric acid, and from the appearance of the crystals under the microscope appeared to be  $\beta$ -cholestanol. The quantity was too small, however, for satisfactory

identification. It was, however, probably  $\beta$ -cholestanol, as the substance was found in considerable quantity in the oily residue left after the crystallisation of the crude coprosterol. Since this work was commenced Windaus and Uibrig [1915] have obtained from crude coprosterol by repeated crystallisation from light petroleum a small amount of a more difficultly soluble substance of higher melting point than coprosterol. The melting point was, however, not sharp. This substance was found to be impure  $\beta$ -cholestanol, which they succeeded in separating from coprosterol by the digitonin method and characterising by means of its esters.

*Examination of the dark oil left after separating the bulk of the crystalline sterols.*

This oil, in suitable quantities, was distilled in superheated steam in the apparatus described above, except that in this case the trap flask was kept in a brine bath at a temperature a few degrees above 100°. The whole distilled over leaving only a slight carbonaceous residue. A portion condensed in the brine flask as a stiff oil, but the bulk passed over into the condenser where it formed a solid emulsion, which was forced out of the condenser by the combined action of the condensed water and the pressure of the steam in solid white candles. This solid emulsion persisted for a long time, but after some days generally separated into oil and water. This was more readily effected by means of ether.

The property this oil possesses of forming these stable solid emulsions suggests that the "sterol" portion of the "unsaponifiable matter" may play an important part in the formation of faeces.

The thick viscid oils which collected in the brine flask were crystallised from acetone. These crystalline fractions—*A*—are described later. The oils from the mother liquors were again distilled in superheated steam. The solid emulsions which collected in the receiver were taken up in ether; the ethereal solutions were then dried and the ether distilled off. The oil left was then distilled in a vacuum of about 1 mm. and roughly separated into two fractions. The lower boiling fraction came over on the paraffin bath up to about 160–170°—*B*.

The higher fraction came over on the metal bath, mainly between 200 and 260°, and constituted much the greater portion of the material. It was very difficult to distil, as it was very viscid and if slightly overheated had a tendency to bump and froth over. An ordinary distillation flask proved quite unsuitable, but after trying various forms of apparatus a retort-shaped vessel was found to be efficient, and bumping was prevented by having a spiral platinum wire at the bottom. The retort was immersed in a metal bath, the portion outside the metal being well lagged. In later experiments it was found better to heat the retort in a Lothar Meyer air bath instead of a metal bath. The bath was kept a few degrees above the boiling point of the fluid. The distillation was done very slowly to prevent bubbling over, and was really more of an evapora-

tion than a true distillation. This high boiling distillate—*C*—set on cooling to a clear pale yellow glass with a slight greenish fluorescence. It recalled in appearance some of the high boiling distillates of petroleum.

On long standing it began to crystallise, bacteria-like crystals gradually spreading through the entire mass, which became opaque.

#### *Examination of fraction A.*

The solid matter from acetone crystallised in matted needles, m.p. 92–95°, and was rather like impure coprosterol. On recrystallisation from absolute alcohol, however, glistening plates—rather like cholesterol—separated. The melting point, after recrystallising several times, was raised to 136–137°, but was not sharp and the substance began to soften at a lower temperature.

These crystals contained 5.35 % of moisture; calculated for  $C_{27}H_{46}O + H_2O$ , 4.45 %.

*Analysis of dried material:* 83.807 % C, 12.034 % H; calculated for  $C_{27}H_{46}O$ , 83.94 % C, 11.92 % H; for  $C_{27}H_{48}O$ , 83.505 % C, 12.370 % H.

100 g. of 96 % alcohol dissolved at 21° 0.844 g. of the crystals. The solubility was therefore less than that of cholesterol. The substance was practically inactive to polarised light. On treatment in ethereal solution with a solution of bromine in glacial acetic acid a crystalline precipitate was formed. This was filtered and washed first with strong acetic acid and finally with water. After drying *in vacuo* it melted at 115–116°, and contained 29 % of bromine (calculated for cholesterol dibromide, 29.3 %). The yield was however less than half the theoretical.

The acetate was prepared in the usual manner. It crystallised from alcohol in glistening thin plates or flat needles. It was only slightly soluble in cold but readily in hot alcohol. It melted with previous softening at 105–106°, but on cooling did not solidify again until 80°. The benzoate was made by the pyridine method. After crystallisation from alcohol it began to shrink together on heating at 107° and ran in the tube at 120° to a clear liquid. During the heating it showed a colour display intermediate between those of cholesterol benzoate and  $\beta$ -cholestanol benzoate. After repeated recrystallisation from alcohol the product became more like  $\beta$ -cholestanol benzoate, and on heating coagulated at about 130° with a bright colour display, emerald green, blue and red according to the point of view. The colours vanished sharply at 157°, a clear colourless liquid remaining in the tube. On cooling the colour display appeared again at 157° and continued after the point of solidification. On mixing with some  $\beta$ -cholestanol benzoate the phenomena were unchanged. From these properties the inactive crystals appeared to be a mixture of approximately equal parts of cholesterol and  $\beta$ -cholestanol. In order to prove this the remaining substance was precipitated in ethereal solution with half the theoretical quantity of a solution of bromine in glacial acetic acid that would have been required had the substance consisted only



of cholesterol. After standing in an ice bath the crystals were filtered on the pump. The ethereal filtrate was poured into water and the solid recrystallised twice from alcohol. It was identified as  $\beta$ -cholestanol by mixed melting and the properties of its acetate and benzoate.

The dibromide was reduced by zinc dust and glacial acetic acid, and the product after hydrolysis with alcoholic potash was recrystallised from alcohol. It consisted of pure cholesterol.

This conclusion was also confirmed by the Liebermann reaction.  $\beta$ -Cholestanol does not give any colour reaction with acetic anhydride and a drop of sulphuric acid, or at any rate reacts only slowly and very slightly under the conditions in which cholesterol gives its typical reaction. The cholesterol was therefore estimated in the crystals by the method of Grigaut [1913] and they were found to contain 63.01 %.

#### *Examination of fraction B.*

This fraction consisted of a yellowish waxy solid, which separated from acetone as a white wax smelling of new mown hay. It was very volatile in superheated steam and readily distilled *in vacuo*. The material was submitted to an elaborate fractionation in a high vacuum. This proved rather difficult as it was not easy to keep the pressure quite constant. Ultimately the following fractions were obtained at about 1 mm.: (1) 112–118° (4 cc.); (2) 120–140° (3.75 cc.); (3) 140–150° (about 5.5 cc. mostly round 145°); (4) 155–165° (3 cc.); and about 6 cc. of oil above 200°, which was added to the main fraction C.

The first fraction was a white wax which, on treatment in chloroform solution with acetic anhydride and a drop or two of sulphuric acid, gave a reddish brown solution. The colour deepened on standing, and was very similar to the colour obtained with ferric chloride and an acetate. No change in colour took place on standing. The fraction 140–150° behaved similarly, but the higher fractions gave the sterol colour changes, *i.e.* the reddish brown gradually changed to green.

As it did not prove possible by distillation, at any rate with the quantities of material available, to isolate any pure products, the low boiling fractions which came over at 100–140° according to the pressure were dissolved together in a few cc. of alcohol, and the strong solution cooled in a good freezing mixture. With a suitable amount of alcohol the solution set to a sloppy crystalline mass. If it set too solid more alcohol was added. The crystals were then filtered by means of the pump through a well cooled filter. The crystalline matter on the filter was then recrystallised from acetone in which it was rather less soluble than in alcohol. It crystallised in leaf-like crystals which melted at 49–50°. The mother liquors on standing *in vacuo* set to a crystalline mass mixed with oil. This was freed from oil by spreading on porous tile, and on recrystallisation from alcohol a further crop of the above substance was obtained. In this manner several grams of crystals, m.p. 49–50°, were isolated. They proved

to be cetyl alcohol, and on mixing with a pure specimen of this substance showed no reduction in melting point.

*Analysis:* 79.31 % C, 14.05 % H; calculated for  $C_{16}H_{34}O$ , 79.34 % C, 14.05 % H.

On oxidation in glacial acetic solution by means of chromic acid, and also by heating to 250° with potash lime, it yielded palmitic acid, m.p. 62–63°.

0.1849 g. required for neutralisation 7.03 cc. *N*/10 soda; calculated for palmitic acid, 7.23.

The silver salt contained 29.15 % Ag; calculated for  $C_{16}H_{31}O_2Ag$ , 29.71 %.

A small amount of crystalline matter, m.p. 55–56°, was also isolated, but in too small quantity for purification. It was evidently a mixture of cetyl alcohol with some higher alcohol, and on oxidation yielded a mixture of acids.

#### *Examination of fraction C.*

This was separated into a number of fractions by distillation under a pressure of about 1 mm., but several on standing became opaque through formation of crystals, and evidently contained some coprosterol or other bihydrocholesterol. The various fractions were therefore dissolved in alcohol and separately precipitated by an excess of an alcoholic solution of digitonin. After standing the alcohol was evaporated at a low temperature and the oil separated from the insoluble digitonides and excess of digitonin by means of ether. After distilling off the ether the oils were again submitted to an elaborate process of fractionation in a vacuum of about 1 mm. This operation, particularly in the case of the higher boiling portions, was difficult and tedious. The difficulty in getting apparatus made during the years 1915–1919 rendered it necessary to work on a small scale, and to work up the material in comparatively small portions. The oils were also very viscous and showed a marked tendency to froth over, so that it was necessary to carry out the distillation very slowly and keep the temperature of the metal or air bath only a few degrees above the temperature of the distilling fluid. It was also found desirable to have the retort completely immersed in the bath. The process was therefore more of the nature of an evaporation than an ordinary distillation. It was also found difficult to keep the pressure constant within a millimetre or two with the pump available. Small variations at these low pressures have an appreciable effect on the boiling point. The apparent temperatures of distillation seemed also to be affected by the temperature of the bath.

Eventually the following fractions were obtained:

(1) 134–140°. This was a waxy material which was found to be similar to the cetyl wax described under *B*.

(2) Small intermediate fractions—155–165°, 165–175° and 175–200°.

(3) The main bulk distilled between 198–210°. It was obtained in various experiments in a series of fractions—198–200°, 202–204°, 199–202°, 202–210°,

200–205°. These were evidently the same substance, the differences in temperature range being probably due to slight unavoidable differences in conditions during the different distillations.

(4) A higher fraction, smaller in quantity—215–225°.

The fractions (3) and (4) all came over as pale yellow clear oils, having a faint greenish fluorescence.

They set on cooling to a clear glass, melting at 16–18°, and gave the sterol colour reactions with acetic anhydride and a little sulphuric acid in a well-marked though modified manner.

On analysis the various fractions gave the following results:

		% C	% H
No. 2.	155–165°. Thick oil ...	82.13	12.83
	165–175° ... ..	83.03	12.17
No. 3.	198–210° ... ..	84.81	12.19
	200–206° ... ..	85.48	12.40
	199–202° ... ..	84.49	12.40
	202–210° ... ..	83.70	12.36
No. 4.	215–225° ... ..	82.97	12.15

Determinations of molecular weight by depression of the freezing point of benzene, in the case of a fraction 200–206°, gave values of 355 and 395; calculated for  $C_{27}H_{48}O$ , 388.

In the case of substances of such high molecular weight and high carbon content, it is difficult to select a formula from the results of combustion. The figures for the fraction 202–210°, however, agree closely with those for cholesterol and bihydrocholesterol.

	% C	% H
Calculated for $C_{27}H_{46}O$ ...	83.94	11.92
„ $C_{27}H_{48}O$ ...	83.51	12.37

This particular fraction remained as a transparent glass for several years. The other glassy fractions, however, on standing for some months gradually became more or less opaque owing to gradual separation of crystalline matter, and were very similar in appearance to bacterial growths in gelatin.

From these results it seemed probable that the fractions above 200° consisted of an amorphous substance of the sterol group mixed with more or less of some hydrocarbon or wax of very high molecular weight.

Attempts to separate crystalline matter by crystallisation from solvents in the ordinary manner were unsuccessful, but it was found possible to separate small quantities of crystalline matter by cooling strong solutions in acetone in a freezing mixture. All the fractions were therefore repeatedly treated in this manner. The waxy solids were too small in quantity for further treatment or identification, but the oils left after evaporating the acetone remained clear on long standing. They were redistilled in a vacuum of about 1 mm., and came over between 200–230° according to the pressure. They were all colourless pale yellow oils which set to glass on cooling. This glass melted about 16–17° to a very viscid oil.

On analysis the following results were obtained:

	% C	% H
(1) 212–230° (1 mm.) ...	83·12	12·56
(2) 214–218° „ ...	83·11	12·19
(3) 202–216° „ ...	84·04	12·18
(4) 204–220° „ ...	83·45	12·09
(5) 214–225° „ ...	84·16	12·21
(6) 230–240° (2 mm.) ...	83·75	12·01
(7) 215–224° (1 mm.) ...	84·04	12·23

From the variations in boiling point and in the figures\*by combustion it is evident that this oil is not a pure substance. It is impossible to deduce a formula from the figures. They might indicate anything from  $C_{24}H_{42}O$ , which requires 83·24 % C, 12·14 % H, to  $C_{32}H_{58}O$ , 83·84 % C, 12·66 % H, or, if the substance is unsaturated, from  $C_{24}H_{40}O$ , 83·72 % C, 11·63 % H to  $C_{32}H_{56}O$ , 84·21 % C, 12·28 % H.

From the boiling point, the colour reactions and great stability, it would seem probable that it may be a sterol of formula  $C_{27}H_{48}O$  or  $C_{27}H_{46}O$ , or a mixture of a saturated and an unsaturated sterol.

Further details of the properties of this amorphous substance are given later on in this paper.

The digitonin precipitates, obtained from various portions of fraction *C*, were dissociated in vapour of boiling xylene, and the solid matter from the xylene was fractionally crystallised from acetone.

In this way, two fractions, one of indeterminate crystalline matter and the other in form of flat needles or spangles, were obtained.

The indeterminate crystals under the microscope appeared to consist of plates similar to crystals of  $\beta$ -cholestanol.

On heating in a capillary tube they began to soften at 122° and melted at 130–131°. The acetate of this substance was prepared in the usual way and crystallised from alcohol in shining leaflets, which on heating in a capillary tube began to soften at 120° and melted at about 124°, but the melting point was not sharp.

The benzoate was made by the pyridine method and after several recrystallisations from alcohol, in which it was difficultly soluble, showed the melting point and characteristic colour display of  $\beta$ -cholestanol benzoate. A mixed melting with  $\beta$ -cholestanol benzoate showed no depression of melting point, but rather more brilliant colours. Evidently the indeterminate crystals were mainly  $\beta$ -cholestanol. The needle-shaped crystals under the lens were very like phytosterol. They began to soften at 132° and melted at 136–137°. The acetate crystallised from alcohol in thin glistening leaflets, which on heating began to soften at 135° and melted sharply at 138–139°. The melted mass on cooling slowly crystallised in needle form. The substance did not form a benzoate by the pyridine method. The quantity was too small for further characterisation, but it was probably a phytosterol derived from vegetable food.

The results detailed above were obtained with mixed faeces—adults and children.

In the examination of the metabolism of a patient, suffering from anorexia nervosa, who was supposed to be on a specified diet, an extraordinarily large quantity of unsaponifiable matter was found in the faeces—40–50 g. in a week. This was distilled in a vacuum of 1 mm., and divided into a series of fractions ranging from 100–210°. These proved to be mainly paraffin hydrocarbons, and subsequent inquiry revealed the fact that owing to a misunderstanding the patient had been dosed with liquid paraffin up to the day on which the dietetic experiment commenced.

This result suggested that possibly the mixed faeces examined might have contained a certain amount of this material, which would contaminate the intermediate and higher fractions, and might account in the latter for the higher carbon and hydrogen content than that required for a true sterol.

It was therefore thought desirable to examine faeces from adults on rigid diet, and also from infants.

*Experiments with adults on known diet.*

For this purpose use was made of material accumulated in a series of experiments, on the digestibility of breads made from different kinds of flour, for a Report by the Food (War) Committee of the Royal Society [1918]. The subjects experimented on were for the most part laboratory attendants and assistants. The pre-arranged daily diet consisted of 800–1000 g. bread, 50 g. butter, 50 g. cheese, 50 g. minced meat, 100 g. of fruit jelly, 600 cc. milk, 30 g. sugar and tea or coffee *ad lib.*, though some individual minor variations were allowed. The subjects were fed for periods of ten or eleven days and the faeces of the last six used. The dried faeces, finely powdered, were extracted with ether, and the extract saponified in the manner already described.

The unsaponifiable matter was then crystallised from acetone as before to separate oil and crystalline matter as far as possible. In this case, however, the remaining oil was treated in alcohol solution with excess of digitonin to get rid of the remaining precipitable sterols before distillation.

The oil was then distilled in superheated steam, and the distillate fractionated in a high vacuum.

The oil was somewhat greater in quantity than the crystalline sterols. About 9 g. on distillation in superheated steam only left in the distilling flask 0.14 g. of carbonaceous matter.

The distillate proved rather difficult to fractionate in a high vacuum as traces of some decomposable matter were present and rendered it difficult to keep the pressure at 1 mm. In the initial distillation the following fractions were obtained:

- (1) Up to 167° at 2–3 mm., but mostly round 127°. Wt. 0.49 g.  
This was liquid and on standing deposited crystals.
- (2) 177–187° at 2–3 mm. This was solid wax. Wt. 0.87 g.
- (3) 187–197° at 5 mm. Wt. 0.68 g.
- (4) To 225° at 5–6 mm. Wt. 0.83 g.
- (5) 227–287° at 2–3 mm. Wt. 5.69 g.  
This was a yellowish oil with a greenish fluorescence, which solidified to glass on cooling.

Residue left in flask, 0.38 g.

These fractions, beginning with No. 2, were now refractionated, and no difficulty was found in keeping the pressure at 1 mm. The following fractions were obtained:

(a)	Up to 137° at 1 mm.	—	This solidified in the receiver as a white crystalline wax.
(b)	130–147°	„	— This was solid, and did not give any colour with acetic anhydride and sulphuric acid.
(c)	149–180°	„	— A viscid oil.
(d)	187–211°	„	Wt. 1.79 g. This solidified to a transparent glass, which changed to a viscid pale yellow liquid at 20–22°.
(e)	211–221°	„	Wt. 2.58 g. This was a pale yellow oil with a greenish fluorescence, which set to a glass on cooling.
(f)	227–257°	„	Wt. 0.72 g. A clear oil with a greenish fluorescence, especially marked in chloroform solution.

Fractions (a), (b) and (c) were dissolved in a little acetone and frozen. The solution set solid, but on filtering on the pump only a small amount remained unmelted. This was recrystallised from alcohol and identified as cetyl alcohol. From the acetone mother liquors a portion of a liquid nature was obtained. On heating this with potash lime hydrogen was evolved; 0.8 g. mixed with four times its weight of potash lime and heated at 230–250° gave 75 cc. of hydrogen at 25° and 760 mm. The residue was dissolved in hydrochloric acid and the fatty acids extracted with ether. The ethereal solution was extracted with aqueous potash, and the alkali solution again acidified and extracted with ether. On evaporating the ether a brown greasy acid was obtained. This was purified by distillation *in vacuo*, and proved to be a mixture of crystalline and oily acids, but too small in quantity for further separation. Evidently, however, the fractions (a), (b) and (c) contained cetyl and other alcohols of the paraffin series. The fractions (d), (e) and (f) all gave the Burchardt-Liebermann reaction in a modified though well-marked manner, and were quite similar to the corresponding fractions from the mixed faeces.

<i>Analysis:</i>	% C	% H
(d)	84.61	11.93
(e)	83.48	11.95
(f)	83.61	11.64

The crystalline sterols proved to be mainly coprosterol with a small amount of  $\beta$ -cholestanol.

The results of this investigation were therefore very similar to those obtained using mixed faeces. In 27 diet periods, during which four different kinds of bread were used, the rest of the diet being much the same, the ratio of the average amount of sterols precipitable by digitonin to unsaponifiable matter not so precipitated was 1 : 0.734. The ratios in individual cases were variable, ranging from 1 : 0.25 to 1 : 1.18, but in the majority of cases the individual ratios were not far from the mean value.

The ratio of the lower boiling portion, which did not give the Burchardt-Liebermann reaction, to the high boiling "sterol" portion which did, was about 1 : 4.

*Examination of infants' faeces.*

The faeces of healthy infants were examined in a similar manner.

In the case of breast fed healthy infants in the early weeks of life the crystalline sterol matter was found to consist entirely of cholesterol. The portion of the unsaponifiable matter not precipitated by digitonin was very variable in quantity in different individuals, and was usually considerably in excess of the cholesterol. This non-cholesterol matter has not yet been thoroughly examined, owing to the difficulty in obtaining a sufficient supply of digitonin, but it was found that in nearly all cases, after completely separating cholesterol by means of digitonin, the oil washed away from the insoluble digitonide by means of ether or light petroleum no longer gave either the normal or modified reaction with acetic anhydride and sulphuric acid. On treatment in chloroform solution with excess of acetic anhydride and a few drops of sulphuric acid a reddish brown colour developed but this did not change, and even on long standing no trace of green colour was noticed. On allowing to stand until the chloroform had evaporated spontaneously crystalline matter separated which could be isolated after dilution with water. Possibly this may afford a convenient method of purifying and identifying some of the constituents. A considerable amount of material from infants of various ages is being accumulated, and we hope to deal with this in another communication.

On the other hand, in pathological cases the amount of cholesterol is often less and the accompanying oils much greater in quantity than normal, and the latter show the acetic anhydride sulphuric colour reaction in the modified manner observed in the case of adult faeces. As an example we may quote the case of a prematurely born child (7 months), the fat metabolism of which was examined in some detail. It was brought into the hospital in the summer of 1915, when a few weeks old, suffering from bilateral conjunctivitis, and remained several months. Very little history of the case is available, but it was stated that its weight at birth was  $3\frac{1}{2}$  lbs., and during its stay in hospital the stools according to the reports were apparently normal. After leaving the hospital the case was lost sight of, but subsequent inquiry elicited the information that "nothing of interest" developed and the infant gradually wasted away and died at the beginning of 1916, but no post mortem was made. During its stay in hospital a feeding experiment was done for seven days. It was fed on Allen and Hanbury's Infant Food No. 1, and weighed during the period 5.5 to 5.75 lbs. During the seven days it consumed 627 g. of the dry food, and excreted far more fat, measured as ether extract, than it ingested. The intake of unsaponifiable matter was 1.68 g. containing 0.36 g. of cholesterol, and the output in the faeces 25.5 g. of unsaponifiable matter containing 3.65 g. of cholesterol. The excess of output over intake was thus 23.8 g. of unsaponifiable matter and 3.29 g. of cholesterol, *i.e.* 3.4 g. and 0.47 g. respectively per day. The oil left after separation of the cholesterol digitonide gave the acetic anhydride sulphuric reaction in a well-marked manner.

The main bulk of the unsaponifiable matter was extracted first with acetone and then with alcohol, and the solutions allowed to deposit as much crystalline matter as possible. A small portion was insoluble or difficultly soluble in alcohol. This was dissolved in benzene and the solution diluted with ethyl acetate and left to crystallise. The mother liquors after separating as much crystalline matter as possible were evaporated and distilled in superheated steam. The great bulk distilled over as a solid emulsion, in the manner already described, leaving only a very small amount of carbonaceous residue. The distillate after separation from water was taken up in acetone and the solvent allowed to evaporate spontaneously, when the material gradually separated in oily form. It was collected in this manner in four fractions. Each fraction was then dissolved in acetone to a fairly concentrated solution and cooled in a good freezing mixture. In each case a small amount of wax solid at ordinary temperatures, a portion solid in the cold but fluid at laboratory temperature and an oil were obtained. These portions were then separately submitted to fractional distillation at a pressure of 1 mm. The fractions of similar range from different specimens were mixed and refractionated. In many cases the fractions were further purified by again freezing from acetone. In this manner the material was separated into (a) crystalline waxy solids, (b) a series of fractions which gave no colour with acetic anhydride and sulphuric acid, and (c) a series which gave with this reagent a reddish brown colour which more or less rapidly changed to bright green.

*Crystalline fractions (a).* From this crystalline matter about 1 g. of a substance insoluble in cold alcohol but soluble in hot was isolated. It separated from hot alcohol in gelatinous form. It was difficultly soluble in acetone but readily in benzene and in chloroform. It was readily crystallisable from benzene solution on addition of acetone. It melted sharply at 68–69°, and on cooling resolidified again at 67°, and remelted again at 68–69°. It was quite stable at 200° and could be distilled. It gave no trace of colour with acetic anhydride and a drop of sulphuric acid.

<i>Analysis:</i>	% C	% H
	82.52	13.90
	82.79	13.73
C <sub>30</sub> H <sub>60</sub> O requires	82.57	13.76
C <sub>31</sub> H <sub>62</sub> O „	82.66	13.78
C <sub>32</sub> H <sub>64</sub> O „	82.76	13.79

Cholesterol was also isolated and identified by its crystalline form, melting point, dibromide and characteristic benzoate.

The white waxy materials, isolated from the fractions (b) by freezing from acetone, after recrystallisation from alcohol melted at various temperatures between 50 and 70°, but were too small in quantity for further separation and identification. One fraction, however, which melted at 49–50° was recognised as cetyl alcohol.

*Fractions of group (b) which did not give the sterol colour reaction.* These were (1) 190–198° at 1 mm., weight 0.37 g., (2) 198–204° at 1 mm. wt. 0.32 g.,



(3) 182–190° at 1 mm. wt. 0.52 g., (4) 190–212° at 1 mm. wt. 0.54 g., (5) 212–224° at 1 mm. wt. 0.33 g., (6) 208–230° at 1 mm. wt. 0.32 g.

These were all low melting white waxes, soluble in acetone, from which they separated in solid form on freezing.

No. (3) gave on analysis 85.62 % C, 13.54 % H.

This analysis, which adds up to 99.2, suggests a mixture of hydrocarbon and alcohol. Possibly the infant might have been dosed with liquid paraffin, but there was no mention of this in the records of the case.

A portion of these waxes was dissolved in glacial acetic acid and oxidised on the water bath with the requisite amount of chromic acid for one hour. The product was poured into water, the precipitate taken up in ether and the acids formed isolated in the usual manner. The product appeared to be a mixture of acids, but was too small in quantity for any attempt to separate and identify them. Titration in alcoholic solution gave an average molecular weight of 290.

Group (c), which gave the "sterol" colour reaction, contained the following fractions: (7) 167–169° at 1 mm. wt. 0.85 g., (8) 185–195° at 1 mm. wt. 1 g., (9) 192–208° at 1 mm. wt. 0.08 g., (10) 209–220° at 1 mm. wt. 0.5 g., (11) 207–210° at 1 mm. wt. 0.16 g., (12) 218–220° at 1 mm. wt. 0.43 g., (13) 212–230° at 1 mm. wt. 0.97 g., (14) 205–213° at 1 mm. wt. 0.4 g.

These all remained quite transparent on standing many months. Some other intermediate fractions however deposited crystals on standing, and these were separated and identified as cholesterol.

Fractions (7) and (8) differed from the rest in being at ordinary temperatures quite mobile yellow oils.

Fractions (7) and (8) were analysed:

	% C	% H
(7)	83.16	12.36
(8)	84.89	12.60

Both appeared to be unsaturated. No. (7) had an iodine value 49.0 (Wijs).

Both gave on treatment in chloroform solution with acetic anhydride and a drop or two of sulphuric acid a reddish brown colour, which in two or three minutes turned a dusky sage green and then bright green. This faded on standing over night to a brown.

As there might have been esters that had escaped hydrolysis in the original saponification, they were heated for some hours in sealed tubes with alcoholic potash at 110–120°. On cooling the material was recovered quantitatively and apparently unchanged. No acid was found on examining the alkaline mother liquors. On treating the recovered oil with digitonin no trace of precipitate was obtained.

The other fractions were at ordinary temperatures transparent pale yellow glasses, or else very viscous oils which would scarcely flow. They gave the sterol colour reaction in the modified way described above in a well-marked

manner, and appeared to be similar to the amorphous glassy substances obtained from the faeces of adults.

On combustion, fraction (13) gave 84.12 % C, 12.40 % H. Fraction (12) had an iodine value 73 (Wijs), fraction (10) 55.1 and another, 180–190°, 36.6.

*Examination of the faeces of soldiers in training.*

In order to ascertain whether cholesterol as such is excreted by the normal adult along with coprosterol and  $\beta$ -cholestanol, it was necessary to examine a large quantity of the excreta of adults on a more or less known diet.

Through the kindness of the D.D.M.S. of the Eastern Command and particularly of Lt.-Col. Jacomb Hood, I was able to obtain 51 stools of soldiers in training. They had the ordinary military ration of 1915, and were actively engaged in military duties.

This material yielded, on extraction in the usual manner, 110 g. of ether extract. This contained a considerable amount of free acid and required for neutralisation 7.23 g. of caustic soda, equivalent to about 51 g. of stearic acid. After saponification in the manner already described it yielded 26.93 g. of unsaponifiable matter as a dark brown oil. 0.6733 g. of this oil on precipitation with digitonin in alcoholic solution gave 1.1452 of compound equivalent to 0.2783 g. of sterol reckoned as coprosterol. The ratio of sterol precipitable by digitonin to the rest of the unsaponifiable matter was therefore 1 : 1.42, and the total unsaponifiable matter contained 11.13 g. of crystalline sterols—mainly coprosterol. The rest of the unsaponifiable matter was then treated with hot acetone, when it readily dissolved with the exception of a small amount of an insoluble crystalline substance. This weighed 0.6735 g. It was difficultly soluble in hot acetone, but soluble in boiling alcohol, from which it separated on cooling in a gelatinous form. It was readily soluble in benzene and chloroform, and could be crystallised from a mixture of benzene and ethyl acetate. It melted at 80–81°, and gave no colour with acetic anhydride and sulphuric acid. It was very similar to the substance isolated from the early born infant described above, but had a much higher melting point.

<i>Analysis:</i>	% C	% H
	82.53	14.68
	82.12	14.16
Calculated for $C_{30}H_{68}O$	82.19	14.15

It seemed similar to the descriptions of myricyl alcohol. The acetone solution of the oil on standing deposited a rich crop of crystals of coprosterol, from which 9.1 g. of pure substance were obtained. The mother liquors by the digitonin method gave about 5 g. of insoluble compound which yielded about 1 g. of crystalline sterol. On fractionation from alcohol, 0.5 g. was obtained in the form of glistening leaflets and the rest in nodules of needles, mainly coprosterol. The leaflets were optically inactive, melted with previous softening at about 133° and were very like the similar material obtained from the Yorkshire

faeces. They were found by similar methods to consist of approximately equal parts of cholesterol and  $\beta$ -cholestanol.

Finally an artificial mixture of cholesterol and  $\beta$ -cholestanol in equal proportions was crystallised from alcohol and found to have properties closely resembling these crystals.

After separating the sterol-digtonide, 14 g. of brown oil partially dry was left. This was distilled in superheated steam, when it came over in the usual manner. 4.8 g. were carried over into the receiver in the form of a solid emulsion, 7.07 g. were caught in the trap flask, and only 0.2 g. of tarry residue remained in the distilling flask.

The oil from the emulsion was then distilled at about 1 mm. pressure, and the part distilling below 120° was dissolved in a little alcohol and cooled in a freezing mixture. In this way a small quantity of crystalline matter was obtained, which after purification melted at 49–50° and was identified as cetyl alcohol. The rest separated from acetone on freezing in solid form, but was difficult to filter. It was therefore added to the rest of the oil, which was then fractionated at a pressure of about 1 mm. first from a paraffin bath and then from a metal bath. 0.8 g. passed over below 150°, mostly round 120°; 1.48 g. above 150° on paraffin bath, and 7.5 g. above 200° on metal bath. The residue weighed 0.68 g. It was impossible to keep the pressure constant during this preliminary distillation owing to some slight amount of decomposable matter. On refractionation this difficulty was not encountered. Ultimately the following fractions were obtained at a pressure of about 1 mm.:

(1) 100–109°	Wt 0.2 g.	Liquid which solidified to wax on cooling.
(2) 127–130°	„ 0.3 „	Waxy solid.
(3) 130–134°	„ 0.3 „	„
(4) 136–140°	„ 0.11 „	„
(5) 140–164°	„ 0.66 „	Viscid oil, which partly crystallised on standing.
(6) 164–190°	„ 0.45 „	Clear liquid, solidifying to glass on cooling.
(7) 190–202°	„ lost	
(8) 202–209°	„ 1.4 „	Pale amber coloured clear glass.
(9) 209–230°	„ about 4 g.	This was distilled very slowly, and the temperature recorded by the thermometer seemed to vary with the temperature of the bath. It was an amber coloured glass at ordinary temperature.

A small amber coloured residue was left in the flask.

The fractions (1) to (5) all gave in chloroform solution with acetic anhydride and a drop or two of sulphuric acid a reddish brown colour, which did not change on standing. The fractions (7) to (9) and also the residue on similar treatment gave a reddish brown colour which changed in a minute or two to a dusky green, and finally to a bright green. On standing over night this green gradually faded and became brown. Thus approximately about one fifth of the oil volatile in superheated steam consisted of alcohols which did not give sterol colours with acetic anhydride, *i.e.* about 8–9 % of the original unsaponifiable matter. Adding also the cetyl alcohol, this non-sterol portion would amount to about 11–12 % of the total unsaponifiable matter.

Fractions (8) and (9) were analysed and found to contain:

	% C	% H
(8)	84.29	12.10
(9)	84.73	11.95

*Properties of the amorphous glass distilling at 200–230°.*

The various fractions distilling at this range consisted of pale yellow or amber coloured amorphous glassy solids, melting at 16–18° to thick viscid liquids usually showing in bulk a slight greenish fluorescence.

The different distillates were not pure substances as is evident from the combustions. They appear to contain oxygen in a not very reactive form, and are marked by considerable stability. They give the sterol colour reactions in a modified way. When cholesterol in chloroform solution is treated with excess of acetic anhydride and a few drops of sulphuric acid, in general a pink colour first appears which rapidly changes to mauve, then violet, blue, blue green and finally green. The green stage is persistent for a long time, but gradually fades and after some hours a brown solution is left. With less quantities of sulphuric acid the initial pink and mauve stages often do not appear. Coprosterol under similar treatment gives a slate blue which deepens to blue and then passes through blue-green to a green similar to that of cholesterol. This slowly fades in a similar manner.  $\psi$ -Coprosterol gives much less marked colours and they take longer to appear. Generally a slate tint first appears and this changes to green, but the green is of a yellower type than that of cholesterol.  $\beta$ -Cholestanol when pure does not seem to give any colour reaction.

The amorphous bodies behave somewhat differently; on adding the sulphuric acid a deep reddish brown colour first appears, very similar to the colour of ferric acetate. In a few minutes this becomes a dusky sage green, which finally changes to a bright green. This stage persists for a long time. On standing some hours the green gradually fades and gives place to a brown. This green colour, and also that of  $\psi$ -coprosterol, is of a somewhat different quality to that given by cholesterol. The latter is a bluish type of green, the former a yellower type. This renders it rather difficult to compare the depth of colour of the one green with the other in a colorimeter.

The substance appeared to be a mixture of saturated and unsaturated sterols. Determinations were made of the iodine values of various samples by Wijs's method, but the results were very variable.

Fractions (d), (e) and (f) (p. 257) of the subjects fed on the bread diets were found to have values 62.5, 67.4 and 86.2 respectively. The theoretical value for cholesterol is 65.8. In case of specimens from the Yorkshire faeces (p. 255) lower values were obtained—No. 2, 31.1; No. 6, 29.7; No. 7, 43.1.

Other fractions gave 30.8, 30.8, 44.2, 31.1, 37.7, 57, 29.7 and 87.6.

Two specimens from infants' faeces gave 36.5 and 33.3. The substance obtained in the preparation of  $\psi$ -coprosterol (p. 248) had a value 37.5.

Much reliance cannot, however, be placed on these values since it has been shown by Smedley MacLean and Thomas [1920] that, for reasons not yet elucidated, Wijs's method gives abnormally high and variable values for cholesterol. This observation we can fully confirm.

Attempts to prepare crystalline or solid bromine addition products yielded negative results.

Attempts to determine the function of the oxygen in the substance also yielded inconclusive results. It had no reaction with hydroxylamine and other ketone reagents.

All attempts to prepare crystalline esters failed, though some indications that the substance was of an alcoholic nature—possibly tertiary—were obtained.

A specimen, B.P. 202–206°, was acetylated by means of acetic anhydride and sodium acetate and yielded a clear viscid oil. This could not be crystallised, but distilled in a high vacuum between 230–240° without apparent decomposition. With the Burchardt-Liebermann reagent it gave a reddish brown colour, which rapidly turned dusky green. On hydrolysis it yielded 7.46 % acetic acid; calculated for cholesteryl acetate 14.02 %. The unsaponifiable matter in the above experiment was recovered and again acetylated by prolonged boiling with acetic anhydride and sodium acetate. The product was not distilled, but freed from excess of acid by shaking the ethereal solution with alkali. On hydrolysis it now yielded 14.45 % acetic acid. Apparently therefore the substance had been acetylated.

On boiling with chloroacetyl chloride, hydrogen chloride was evolved, but the product was non-crystalline, and attempts to prepare a solid body by replacing the chlorine by a piperidyl residue failed. An amorphous product obtained by benzoylating a specimen with benzoyl chloride in pyridine solution at the boiling point, after being thoroughly freed from any free benzoic acid, was distilled in a high vacuum. It decomposed giving benzoic acid and an oil, the main portion of which distilled at 200–220° at 1 mm. This was a viscid amber oil at summer temperature which set to a glass on cooling, and gave the sterol colour reaction markedly in the modified manner. It contained 86.04 % C and 11.53 % H. Attempts to isolate a crystalline hydrocarbon from this, however, failed.

Other specimens gave analogous results, and probably the material consisted of an alcohol or mixture of saturated and unsaturated alcohols which was partially benzoylated under the conditions of experiment, and on distillation the benzoate decomposed into benzoic acid and a hydrocarbon, which was obtained in the higher fraction of the distillate along with original unchanged material.

In order to ascertain whether it would be possible to isomerise the material in a manner similar to the change of coprosterol to  $\psi$ -coprosterol and *vice versa*, and so possibly obtain a crystalline substance, 2 g. of a specimen, which distilled at about 200° and was a clear glass melting at 16–18°, were boiled with

20 cc. of amyl alcohol and 1 g. of sodium for seven hours. The amylic solution, after cooling, was washed first with water and then with acid. The amyl alcohol was then driven off in steam and the residue taken up in ether. Attempts to crystallise the substance from various solvents failed. The solutions on spontaneous evaporation all left a clear glass. It was therefore distilled at a pressure of 1 mm. with the bath only a little higher than the apparent temperature of distillation. It began to distil at  $200^{\circ}$ , passed over at much the same range as the original, and solidified in the receiver as a pale yellowish glass. It differed from the original in giving the sterol colour reactions in a typical well-marked manner—crimson, violet, blue and finally bright green.

*Analysis:* 84.42 % C, 12.26 % H.

The iodine value (Wijs) was found to be 30.5. Calculated for a compound of the carbon content found and one double link = 55.5 %.

On treatment with phosphorus pentachloride the oxygen was replaced by chlorine with evolution of some hydrogen chloride.

In one experiment 0.5 g. of the last fraction of the soldiers' material was dissolved in light petroleum and treated with phosphorus pentachloride in slight excess. The mixture was boiled under a reflux until everything went into solution and evolution of hydrogen chloride ceased. The light petroleum solution was then poured on to a large bulk of water and allowed to stand until the petroleum had all spontaneously evaporated. The water was poured off from the oil, which was again taken up in light petroleum, and the solution well washed with water, dried and evaporated at the ordinary temperature. In this way a thick oil was obtained with a chlorinaceous smell. As it seemed to darken when placed in the water oven it was dried *in vacuo* over sulphuric acid for several days and obtained as a pale yellowish brown oil.

*Analysis:* 11.41 % Cl.

A compound of the formula  $C_{27}H_{47}Cl$  would require 8.76 %;  $C_{24}H_{40}Cl$ , 9.74 %;  $C_{27}H_{46}Cl_2$ , 16.6 %.

This oil on heating at  $110^{\circ}$  in an air bath gave off a pungent acid substance, probably hydrochloric acid. It was heated until it ceased to smell, taken up in light petroleum, filtered from a slight brown residue and the petroleum evaporated. The brown oil so obtained contained 6.05 % Cl. Another specimen treated in the same manner gave exactly the same result.

A specimen, B.P.  $205-215^{\circ}$ , which had been made from oil not absolutely freed from coprosterol by digitonin was treated in a similar manner. The product was reduced by sodium and alcohol in the hope of obtaining a hydrocarbon. For this purpose the product from 0.8 g. of material was dissolved in boiling alcohol in a flask under a reflux and 5 g. of sodium gradually added. After all the metal had dissolved, water was added. An oil separated but no trace of crystalline matter. This was taken up in ether and the solution thoroughly washed with water and dried. After evaporating the ether an oil was obtained of pretty much the same weight as the original material. It was difficultly soluble in hot alcohol, but readily in acetone, ether and chloroform.

Attempts to crystallise it were unsuccessful and it was therefore distilled at a pressure of about 1 mm. It came over on a metal bath heated to 225–240°, and was obtained as a yellow oil which set on cooling to a transparent glass. No residue remained in the flask.

On analysis it was found to contain 85.42 % C, 11.57 % H. The material, however, still contained traces of chlorine. The experiment was therefore repeated using 2.2 g. of a specimen from the bread subjects, B.P. 220–230°. The chloro-derivative was reduced with 14 g. of sodium. The product isolated still contained traces of chlorine. It was therefore dissolved in 100 cc. of amyl alcohol and again reduced with sodium at the boiling point. Water was then added and after standing over night the amyl alcohol was distilled off in steam. The residual oil was taken up in ether and benzene. It could not be got to crystallise and was therefore distilled in a vacuum of 2 mm. or thereabouts. It slowly evaporated over leaving no residue, the temperature indicated by the thermometer in the vapour being 200–214°.

At the laboratory temperature (16°) the distillate was a clear yellow very viscid oil which could just flow. It was free from chlorine and contained 83.74 % C, 11.89 % H, figures which are much the same as those required for cholesterol. Evidently a hydrocarbon had not been obtained. Possibly the chlorine might have been replaced by an ethoxy-group. A substance of the formula  $C_{27}H_{45}O.C_2H_5$  would contain 84.06 % C, 12.12 % H.

On dissolving in chloroform and adding excess of acetic anhydride and a few drops of sulphuric acid a deep reddish brown colour was produced, which rapidly changed to sage green and finally bright green.

Some of the fractions on combustion showed a rather high carbon content, suggesting possibly the presence of a small amount of some hydrocarbon. Fraction (9) from the soldiers' faeces (p. 263) is a case in point. Attempts, however, to isolate any such substance in a crystalline form failed.

0.8 g. of this particular fraction was dissolved in ether and 5 cc. acetic anhydride and a few drops of sulphuric acid added. The usual green colour developed, which slowly faded to brown. After standing several days nothing of a solid nature separated. The whole was again dissolved in ether, 1 cc. of sulphuric acid added, and the solution left to stand for the ether to evaporate spontaneously. After standing many days no solid deposit separated and a thin black fluid remained. On dilution largely with water it gave a homogeneous solution. On addition of alkali, however, a sticky substance separated in flocks. This was filtered off, but on washing with water appeared to dissolve again to a colloidal solution. On again adding alkali the flocculent precipitate reappeared. This was taken up in ether, dried and on again evaporating the ether a stiff oil was obtained very similar in appearance to the original material.

Wacker [1912] stated that the portion of unsaponifiable matter of the fat of tissues not precipitated by digitonin was unstable to alkali, and when boiled with alcoholic potash gave a "resin" and a "fatty acid."

From the mode of preparation and treatment of these amorphous substances of the faeces it seemed unlikely that they would be affected by alcoholic potash, but it was possible that they might contain traces of stable esters that had perhaps escaped hydrolysis in the ethoxide saponification and subsequent treatment.

A number of the fractions were therefore separately heated in sealed tubes with excess of strong alcoholic soda for several hours at 110°. After cooling the contents of the tubes were diluted with water and extracted with ether.

The oils were in every case recovered quantitatively, and, judging by boiling point, general properties and combustion, apparently unchanged. On treatment with digitonin no weighable quantity of insoluble digitonide was obtained. Evidently therefore these clear transparent fractions were stable to alkalis, and contained no esters, though they may have contained minute traces of cholesterol or coprosterol.

#### *Origin of these amorphous substances.*

Two sources suggest themselves, (1) the substances which accompany cholesterol in the unsaponifiable matter of tissue fat, (2) the bile acids or their derivatives.

(1) The sterols precipitable by digitonin constitute only a portion of the unsaponifiable matter of the fat of tissues and organs. Wacker [1912] describes what he called "Begleitsubstanzen des Cholesterins" in human depot fat. He found that on an average the underskin fat contained 65.8 and the mesenterial fat 61.3 % of this substance.

He describes it as a wax-like body melting at 25–32°, which possesses properties analogous to those of cholesterol. It easily emulsifies with water. It is easily extracted from these emulsions by means of ether, otherwise the emulsions persist for a long time. It is soluble in most organic solvents—alcohol, ether, light petroleum, glacial acetic acid—but cannot be made to crystallise. He also states that this "Begleitsubstanz" gives in chloroform solution with acetic anhydride and sulphuric acid a brownish red coloration, but does not mention any further changes in colour.

It was unchanged by acetic anhydride and not precipitated by digitonin.

As mentioned above he states that this wax was unstable to alkalis, and if boiled with sodium ethoxide or alcoholic potash on the water bath there separated after dilution with water a brown resin. The alkaline filtrate on acidification gave "apparently a fatty acid." He does not give any indication of the quantity or nature of this acid.

This description we are able to confirm. A considerable quantity of human omental fat was hydrolysed and the unsaponifiable matter precipitated by digitonin. The oil separated by means of ether from the digitonin precipitate was in the cold of a waxy nature, and when treated in chloroform solution with acetic anhydride and sulphuric acid gave a brownish red colour, which



after a few minutes developed a greenish fluorescence. This became more marked on standing, but on long standing faded to brown.

On distillation in superheated steam it volatilised readily, condensing in the condenser in the white candles of solid emulsion described in the case of the faecal oils. Practically no residue remained either in the trap or in the distilling flasks.

Human muscle was also extracted and treated in a similar manner. The oil distilled in superheated steam in the same characteristic fashion.

Both the above distillates were taken up in ether, and the solutions dried and evaporated. In both cases the oil passed over in a high vacuum at 185–200° leaving only a trace of tarry matter.

A considerable amount of material has been accumulated from various sources, and we hoped to be able to purify this and compare it with the faecal wax. We have, however, been as yet unable to complete this work owing to difficulty in obtaining digitonin in sufficient quantity since the war. We hope, however, to investigate the material more fully later on.

In a series of papers published during the past ten years, J. Lipschütz [1908–1914] has put forward the view that in the general cholesterol metabolism the first oxidative attack on cholesterol takes place in the blood stream with the formation of an oxysterol. This substance he has also found in the unsaponifiable matter of the fat of other tissues. The oxysterol he estimates [1913, 1 and 2] by means of a colour reaction, with glacial acetic acid and sulphuric acid, not given by cholesterol itself. As in the case of the Burchardt-Liebermann reaction for cholesterol, this colour change is a progressive reaction, and the last stage is used. This usually takes some time to attain, but can be hastened by the addition of a little 5 % solution of iron chloride in glacial acetic acid [1908, 1, 2 and 3]. He does not compare the colours in an ordinary colorimeter, but uses a comparative spectroscope and compares the spectral intensities of the absorption bands of the chloroform solution of the substance under examination with those of a standard solution of "pure" oxysterol, the stronger solution being diluted until equality of absorption is attained. The oxysterol for his test solutions he makes by the action of benzoyl superoxide on cholesterol. It is a resinous substance, showing no tendency to crystallise. It dissolves in all media except water, but particularly easily in benzene and methyl alcohol. It has no sharp melting point and has no ordinary property which can serve as a criterion of purity. Lipschütz uses the spectral intensity of the bands in the above-mentioned colour reaction for this purpose.

In order to compare this substance with the faecal distillates it was prepared following exactly Lipschütz's instructions [1914]. The various phenomena noticed and the final product agreed exactly with the descriptions given in his paper. He states that oxysterol, free from cholesterol, is precipitated from its solutions by digitonin, similarly to cholesterol, in small white silvery crystals melting with decomposition at 218°. The weight obtained corre-

sponded to 50–53 % of the oxycholesterol used. Most people would, we think, have interpreted this result as indicating that the oxycholesterol used was not a pure substance, but contained 50 % of some sterol precipitable by digitonin. Lipschütz, apparently on account of the spectral properties, does not draw this conclusion, but regards his substance as being only partially precipitable. In these experiments he followed the procedure described by Windaus [1910] for the estimation of cholesterol. His conclusion would only be understandable if the reaction were a reversible one, or if the oxycholesterol-digitonide were to some extent soluble in alcohol. In either case we should expect the percentage of substance precipitated to vary with the conditions of reaction.

In order to test this point the precipitable "oxycholesterol" was estimated by the modified procedure described by Fraser and Gardner [1910] in which the whole of the alcoholic solvent is evaporated before washing away oil and excess of digitonin. In this way it was found that 49.4 % of the material was precipitated, a result practically the same as that given by Lipschütz. The portion not precipitated was washed away from the compound and excess of digitonin by means of ether. In chloroform solution this gave with acetic anhydride and sulphuric acid a reddish brown colour, which did not however change to green.

To compare the oxycholesterol with the faecal waxes it was distilled in superheated steam. A small portion passed over in the form of a white solid emulsion very similar in appearance to that obtained with other sterols, followed by a viscid yellow oil in somewhat larger quantity. Some decomposition appeared to have taken place, as a considerable amount of a non-volatile brown residue remained in the distillation flask.

The emulsion and the oil were separately taken up in ether and the solutions were dried and evaporated. The oil from the emulsion was found to contain 18.5 % of substance precipitable by digitonin, reckoning it as cholesterol. The part which was not precipitated was dissolved out by ether, and in chloroform solution gave with acetic anhydride and sulphuric acid a brownish red colour which in a few minutes changed to a dusky green. It gave no colour when glacial acetic acid was substituted for acetic anhydride, even on long standing.

The second portion of the distillate, which dried to a sticky mass, was found to contain 25.36 % of a sterol precipitable by digitonin. In chloroform solution it gave with acetic anhydride and sulphuric acid a pinkish brown colour which rapidly changed through deep blue to green, a series of changes much more like those given by cholesterol and coprosterol. The oil washed away from the digitonide gave a reddish brown colour which rapidly turned green, but without any trace of a blue stage. On standing the green gradually faded and left a dirty brown fluid. With glacial acetic acid instead of anhydride it gave a reddish colour, which did not change.

Lipschütz's product, whether a single substance or not, was quite different from the amorphous substance from faeces.

(2) Another source that suggests itself for the sterol waxes of the faeces is the bile acids.

It has long been suspected that cholic acid, the most specific bile acid, and the sterols are members of a hydroaromatic class of polycyclic type, and probably possess a close chemical and genetic relation to one another. It is well known that cholesterol is normally eliminated by the liver, and in 1913 Grigaut stated as his own personal opinion that only a small part of the cholesterol of the cells dealt with by the liver is excreted as cholestérol, the major portion appearing in the bile in the form of a product of transformation—cholic acid, so that according to him cholesterol plays an important part in the formation of bile salts and cholic acid represents in the bile the principal product in the elimination of the cholesterol of the organism. It must be admitted however that he produces little convincing evidence for this view.

Cholic acid and its derivatives, however, give rise to colour reactions quite similar to those of cholesterol. Thus Wieland and Weil [1912] found that cholatrienic acid prepared from cholic acid gave the Burchardt-Liebermann test, and Lipschütz a little later pointed out that cholesterol and cholic acid, when boiled with glacial acetic acid, benzoyl superoxide and concentrated sulphuric acid, gave similar highly characteristic colour reactions.

Clear proof of the connection, however, follows from the recent work of Wieland and his co-workers on bile acids and of Windaus and his co-workers on cholesterol.

In 1912 Wieland and Weil obtained, by the distillation of cholic acid *in vacuo*, an unsaturated acid, cholatriencarboxylic acid,  $C_{24}H_{34}O_2$ . This gave on catalytic reduction by hydrogen in presence of palladium a less unsaturated acid,  $C_{24}H_{36}O_2$ , and finally a saturated monobasic acid—cholanolic acid,  $C_{24}H_{40}O_2$ .

In 1919 this same cholanic acid,  $C_{24}H_{40}O_2$ , was prepared by Windaus and Neukirschen [1920] by oxidation in glacial acetic acid by chromic acid of  $\psi$ -cholestan, so that cholic acid and  $\psi$ -cholestan, a derivative of coprosterol, must have the same carbon skeleton.

In order to obtain, if possible, some indication as to whether the oils of faeces, boiling above  $200^\circ$ , belonged to the sterol group with 27 carbon atoms, or to the bile acid group with 24, they were submitted to drastic oxidation, and we may briefly mention the preliminary results.

2.5 g. of a fraction  $200\text{--}211^\circ$  were dissolved in glacial acetic acid on the water bath and 10 g. of chromic acid gradually added. After heating for several hours the mass was slowly distilled. The distillate was made alkaline and again distilled. This distillate contained some acetone which was recognised by the iodiform test and Marsh's test. The residue in the flask after dilution was extracted with ether, and the neutral and acid products separated in the usual way. The acid was a stiff oil which would not crystallise, but on titration was found to have an average molecular weight of 384. The neutral product weighed 1.5 g. and appeared to be unchanged material.

In another experiment 1.4 g. similarly treated gave 0.019 g. of acetone,

estimated by Scott-Wilson's method [1911]. On evaporating the neutral ether solution the fragrant smell often given in sterol oxidations was noticed. The quantity was much too small for any attempt at identification, but it was probably due to the methylisohexyl ketone isolated by Windaus and Resau [1913]. In another experiment the oil was submitted to drastic oxidation by means of fuming nitric acid. It was hoped that the results might give some indication as to whether the wax originated from a sterol of the cholesterol group or from bile derivatives. It has been shown by Bredt that dinitroisopropane is usually formed by the oxidation of substances containing an isopropyl grouping with nitric acid. Windaus [1918] isolated this substance by the energetic oxidation of cholesterol with strong nitric acid. It is not, however, formed when the  $C_{24}$  acid—cholic acid—is oxidised in a similar manner, as this does not possess an isopropyl-group. The three carbon atoms constituting the difference between the carbon skeletons of cholesterol and cholic acid are apparently in the form of such a grouping.

12 g. of wax, 200–202°, were oxidised with 300 cc. of fuming nitric acid in a very long necked flask. The reaction was very vigorous at first and started at the ordinary temperature, quantities of nitrous fumes being evolved.

As soon as the action slackened, the flask was put on the water bath. Finally the liquid was boiled over a gauze for several hours until the evolution of fumes had nearly ceased. The product was then dealt with in the manner described by Windaus [1918]. In order to isolate the volatile products half the solution was distilled off. The distillate was neutralised with caustic soda and again submitted to distillation. In the distillate very small quantities of oily drops were noted, apparently insoluble in water. They appeared to solidify on cooling and had a characteristic smell, but the quantity was much too small for any attempt at identification. The smell was quite consistent with this being dinitroisopropane, but it could not be further identified. In any case the quantity was very minute. The neutral fluid in the distilling flask was then acidified with sulphuric acid until distinctly acid to tropaeolin paper, and again distilled. The strongly acid distillate was boiled with silver carbonate, when a considerable amount of silver separated. This was filtered off and the filtrate evaporated *in vacuo* over sulphuric acid. A considerable quantity, over 1 g., of silver acetate was obtained in needle shaped crystals.

On analysis this gave (1) 63.9 % Ag; (2) 62.44 % Ag; (3) 63.76 % Ag; calculated for silver acetate, 64.65 % Ag.

The main quantity of volatile acid was evidently acetic acid.

The nitric acid liquor, left after separating the volatile acids, was evaporated in a platinum dish with frequent addition of water to get rid of nitric acid. The residue was a brown syrup which showed no sign of crystallising on standing. This was dissolved in water and neutralised with milk of lime, when a white precipitate containing a good deal of calcium oxalate was formed. This was filtered and the filtrate precipitated with lead acetate. The bulky, heavy precipitate formed was filtered off. The acid obtained from this salt was

amorphous. It gave a soluble barium salt containing 39.2 % of barium. It was not further examined, but was perhaps similar to the amorphous acid obtained by Mauthner and Suida [1903] by oxidising cholesterol with nitric acid.

After separating the excess of lead from the filtrate from the lead salt by means of sulphuric acid, the liquid was evaporated to small bulk and extracted thoroughly with ether. After evaporating the ether the extract was placed in a small retort and heated in an air bath to 220°. It was then cooled and slowly distilled *in vacuo*. At first an aqueous distillate was obtained and at a higher temperature an oil passed over which solidified in the receiver. The distillate was dissolved in hot water and treated with baryta in slight excess and boiled. There separated a small quantity of a difficultly soluble barium salt. This was quantitatively decomposed by dilute sulphuric acid, and after filtering off the barium sulphate the filtrate was evaporated to dryness and boiled out with benzene. The insoluble acid was then recrystallised from water. It melted at 185° and was identified as succinic acid. There was not, however, sufficient for a combustion. The filtrate from the barium succinate was acidified with hydrochloric acid and extracted with ether. After evaporating the ether the residue was completely soluble in benzene, and evidently contained no more succinic acid.

The benzene solution was mixed with light petroleum and on standing crystals were deposited. These were recrystallised from these solvents several times and finally from water. The melting point was now constant—149–150°.

The equivalent weight (by titration) was 71.3.

*Analysis:* 47.42 % C, 7.57 % H; calculated for  $C_6H_{10}O_4$ , 49.31 % C, 6.85 % H. The acid was probably adipic acid.

A very small quantity of another acid was obtained from the benzene mother liquors, melting at 118°. On repeated recrystallisation from water the melting point was raised to 127–128°. Titration indicated an equivalent of 80, or if dibasic, a molecular weight of 160. This would fit an acid of the formula  $C_7H_{12}O_4$ .

The mother liquors on evaporation gave an oil which on standing solidified to a wax. Titration gave an equivalent of 83.4. After recrystallisation from water it shrank together at 94° and melted at 101–102°. It was not further examined.

These preliminary experiments were inconclusive as far as the settlement of the alternative origin suggested is concerned.

The oxidation of this substance is, however, being studied in greater detail, and feeding experiments are in progress which it is hoped will throw light both on the constitution and origin.

The writer feels some diffidence in bringing forward so long an account of a substance, obviously not pure, and perhaps a mixture, but his justification for doing so is that whatever its exact nature it undoubtedly belongs to the sterol or polyterpene group, and in any metabolic experiments dealing with the intake and output of sterols must be taken into account.

In conclusion the writer wishes to express his thanks to Mr F. W. Fox for his help in most of the analytical work in this investigation, and to the Government Grant Committee of the Royal Society for help in carrying out this work.

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